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In the United States Patent and Trademark Office

Appn. Number: 10/760,156
Parent Appn. 10/007,489 Filed: 12/05/2001
Applicant: Elizabeth Gay Frayne
Title: Use of a Modified Phosphate for Enhancing the Natural Mutation Rate in Bacteria and Mutating Recombinant DNA Phage Inserts.
Examiner: Nancy Vogel
Art Unit:1636

Assistant Commissioner for Patents
Washington, District of Columbia 20231

RE: Office Action Dated 6/25/08

Sir:

Please note that the applicant has filed paperwork to have the specification of the present application amended as requested by the examiner to indicate that it is a continuation-in-part of the parent application which has already issued now US Patent 7,125,982 and related divisional Application No. 10/679,305. Below is a response to examiner's current objections as well as amendments to the claims to bring them into compliance.

Regarding Claim Rejections -35 USC 112 –Claim 3

The present invention is a relatively straight forward method for enhancing the natural mutation rate of micro-organisms. The method works by simply culturing cells in a modified media containing thio-phosphate. The thio-phosphate is incorporated into the DNA of growing cells resulting in phosphorothioate linkages in genomic DNA. This modification is known to block the activity of enzymes that act on DNA such as restriction enzymes and DNA repair enzymes but the extent of inhibition varies depending on the enzyme in question (see Burgers and Eckstein, 1979). The present invention demonstrates a 200 fold amplification of the mutation rate in E. coli using thio-phosphate containing media. This is 20 fold more than would be expected from inhibiting editing by DNA polymerase alone.

The advantage of the present invention is the non-toxic nature of the mutagen which allows one to grow cells in mutator media for several generations. The more cell divisions a bacterial cell undergoes the more mutations will accumulate as the mutation rate is based on a frequency of ~1 X 10^{-7} nucleotides per cell generation. This idea is a simple application of theory and nothing new. It is also discussed in (Frayne, 2002, American Biotechnology 21:68). In the present invention I demonstrate this application with the use of M13 phage particles (see Example 1). After one round of infection the phage particles have a mutation rate of one in 400. That would be a lot of phage to screen without a selectable marker. However, one can simply re-infect fresh host cells with these phage particles to produce a mutation rate of one mutation per phage DNA molecule. Hence, multiple rounds of infection have permitted the accumulation of mutations in the phage particles. An additional round of infection might produce a higher mutation rate but since the viability starts to drop after the second round it appears that 2 rounds are useful before the reduction in the number of viable phage would limit the utility of the method. This is because as mutations accumulate in the recombinant DNA target site they also accumulate in the phage. Such mutations can be deleterious to the phages viability and reproduction rate.

I also demonstrate the concept of diluting cells to accumulate mutations in Example 2 using yeast cells. The cells are diluted 1:20 and allowed to grow in mutator media. This allows several generations (4-5 generations) before the media is saturated with yeast cells. The density of cells in the mutator media is somewhat less than wildtype but this is likely due to the impact of thio-phosphate on protein synthesis rather than the 10 fold enhanced mutation rate. In the yeast example given, the mutant phenotype was easily selected for using selection plates that enabled the detection of canavanine resistance as a low frequency. event. Classical chemical mutagens used at high enough concentrations result in the loss of viable cells. Too many mutations can result in the loss of viable host cells to the point where the number are too few to analyze. When using mutator strains (Greener, 1996) the host cells accumulate mutations after inoculation into media and being allowed to grow until a maximum density is reached. DNA plasmids can be isolated from mutator strains and analogous to the phage example used to transfect fresh host cells for another round of amplification to enhance the mutation rate. As DNA plasmids are much smaller than the bacterial genome they can undergo more rounds of replication without loss of

being able to replicate in cells upon transfection.

The following is a direct quote from the original specification

> It is useful to gather information about gene specific mutation rates to assess the overall mutation rate for a given strain. To reduce the number of bacteria required for screening one can examine the accumulation of mutations by serial dilution. A single colony will contain ~1 million cells indicating a 20 fold amplification. If this colony is picked, grown, and replated the number of mutations will increase 400 fold (20 X 20) above the mutation rate per generation. By growing cells in mutator medium or agar plates containing thio-phoshate, the number of mutations can be increased further approximately ~200 fold during each growth stage.

Applicant believes the present application provides ample description of the invention encompassed by the modified claim 3 intended to make the description more clear.

A further benefit of the present invention is the ability to apply selective parameters while an organism is being cultivated in mutator media. This may allow for the accumulation of incremental changes desired as in when one is developing continuous traits.

Regarding Claim Rejections -35 USC 112 -Claims 3-5

The examiner has raised questions regarding the general applicability of the present invention for claims 3-5. The applicant has demonstrated in the parent application and the present application the general utility for the use of thio-phosphate in culture media to modify genomic DNA in a variety of organism including bacteria, yeast, and fish. Multiple factors contribute to errors in DNA replication including the error and editing ability of the polymerase complex as well as post-replication DNA repair functions in cells which is well known from the prior art (see Cooper, D. N. and Karwczak (1990) Hum. Genet. 85:55). The fidelity of replication is higher in eukaryotes than prokaryotes owing largely to enhanced post-replication repair mechanisms. In the course of testing the present invention a significant difference was observed between prokaryotic and eukaryotic cell types with regards to how much the mutation frequency was enhanced. A 200 fold increase was observed in bacteria and only a 10 fold increase in yeast. This is most likely explained by the fact that the phosphorothioate modification of genomic DNA does not inhibit the post-replication DNA repair functions as well in eukaryotic cells.

The present invention works in yeast, just not as well as in

bacteria. Practically speaking one could undergo additional rounds of growth and replication to increase the total number of cumulative mutations in yeast. If a single cell is grown to 1 million cells that represents a 20 fold amplification and if done in mutator media or agar that adds an additional 10 fold increase in mutations for a total 200 fold increase in accumulated mutations. If you desire a mutation rate of at least 1 per 1000 yeast cells then additional rounds of amplification are needed up to a 10^5 fold amplification (assuming an initial mutation rate of 1×10^{-8} for yeast). Approximately, 3 rounds of replication starting with an individual cell would be needed to reach the desired frequency of mutations. Since yeast grow fairly rapidly this is not out of the question. Mammalian cells have an even higher fidelity (10 fold) and take much longer to grow than bacteria or yeast. In contrast with bacteria only two rounds of replication would be needed to produce a mutation frequency of one per bacteria.

The present invention relies on the ability to create chemically modified DNA in vivo. Phosphorothioate linkages are known to generally affect the activity of enzymes that normally act on the unmodified substrates. The invention is not limited by an organisms ability to incorporate thio-phosphate. It is limited though for practical purposes by an organism's natural mutation rate and growth parameters. I have shown that incorporation of this analogue can impair proof reading enzymes in both bacteria and yeast in vivo. It has already been shown in vitro that such modifications can impair the DNA editing activity of E. coli DNA polymerase and T4 DNA polymerase. Applicant feels that the examples given provide strong support for the inventions general utility in creating bacterial variants provided that can be readily cultivated in media or agar plates.

Regarding Claim Rejections -35 USC 112 -Claims 3-5-Clarity

Please note the claims 3-5 have been modified to more distinctly describe the essence of the invention. See claims amendment below.

I wish to declare and make a verified statement that the modified claims contain no new matter as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b) or 1.825 (d).

Please under MPEP 707.07(j) the pro se applicant requests that if the Examiner fines patentable subject matter disclosed in this application, but feels that applicant's present claims are not entirely suitable, the Examiner draft one or more allowable claims for the applicant.

Respectfully submitted,

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